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SALINITY EFFECTS ON THE ACTIVITY OF GRANULAR HEMOCYTES OF AMERICAN OYSTERS, *CRASSOSTREA VIRGINICA*

WILLIAM S. FISHER AND ROGER I. E. NEWELL

University of Maryland, Horn Point Environmental Laboratories, P. O. Box 775, Cambridge, Maryland 21613

ABSTRACT

American oysters, *Crassostrea virginica,* from a high-salinity (HS) and a low-salinity (LS) location in the Chesapeake Bay were acclimated to six salinities (6–36 ppt) in the laboratory for 3–4 weeks. After acclimation, hemolymph was drawn from oysters and granular hemocytes were tested *in vitro.* Measurements of time to hemocyte spreading (TTS) and rate of hemocyte locomotion (ROL) were made in six media ranging in salinity from 6–36 ppt. TTS measurements were fastest at the acclimation salinities and slowed with acute rises in salinity. The time to spreading may be a measure of the osmotic adjustment process. Locomotion was dependent on ameboid shape. ROL decreased with acute rises in salinity for both populations, and increased with acute reductions in salinity for all test conditions except HS hemocytes acclimated at 30 and 36 ppt salinity. ROL tested at the acclimation salinities showed no differences between HS oysters (complete acclimation to lower salinities) but LS oyster hemocytes at 30 and 36 ppt were still slower even after 27 days of acclimation. There were a greater number of agranular hemocytes for HS oysters at all salinities. These findings are discussed in relation to osmotic adjustment, ameboid locomotion, acclimation, and disease susceptibility.

INTRODUCTION

Phagocytosis of foreign particles by hemolymph cells is a primary line of defense for American oysters, *Crassostrea virginica* (Cheng, 1975), and the bulk of phagocytic activity is performed by the granular hemocytes (Cheng, 1975; Foley and Cheng, 1975). Hence, proper function of the granular hemocytes may be required for defense against oyster diseases caused by parasites such as *Perkinsus marinum* (Mackin *et al.,* 1950; Perkins, 1969), *Haplosporidium costalis* (Wood and Andrews, 1962), and *H. nelsoni* (Haskin *et al.,* 1966; Sprague, 1978). A link between these diseases, which are commonly called Perkinsus (or Dermo), SSO, and MSX, respectively, is that each occurs as a chronic infestation only in high salinity regions (Mackin *et al.,* 1950; Mackin, 1955; Wood and Andrews, 1962; Andrews, 1964; Haskin and Ford, 1982). In addition, experimental work has demonstrated that established MSX infections can be rejected by oysters when they are transferred from high to low salinities (Sprague *et al.,* 1969; Haskin and Ford, 1982). One possible explanation for this pattern is that high salinity reduces the oysters' defense capacity and leaves them more susceptible to pathogenic parasites.

The hemolymph of marine molluscs, including bivalves, follows the osmotic strength and ionic composition of the ambient water (Hand and Stickle, 1977; Shum-

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Abbreviations: CMFW—calcium and magnesium free water; FCW—full complement water; HS and LS—high and low salinity oyster populations; TTS—time to hemocyte spreading; ROL—rate of hemocyte locomotion.

way, 1977; Gilles, 1979). Thus, the hemocytes are exposed to the full range of salinities, 6–36 ppt (Galtsoff, 1964), experienced by the oyster. The objective of this study was to determine the effect of salinity changes on specific functions of the granular hemocytes of *C. virginica.*

The characteristic activity of granular hemocytes withdrawn from oysters has been described by Bang (1961) and by Foley and Cheng (1972). Chelating agents, such as ethylenediamine tetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA), are often used to inhibit adherence, aggregation, or transformation of locomotory cells. (Noble, 1970; Edds, 1977; Kanungo, 1982; Kenney *et al.,* 1972). Taylor and Condeelis (1979) noted that calcium chelating agents cause both mobile freshwater amoebae and echinoderm eggs to cease movement and to form spherical shapes. Similar techniques were used in this study to inhibit hemocyte activity prior to *in vitro* experimentation.

Measurement of the time required for transformation, introduced here for the first time, is the cell response time, *i.e.,* the time from stimulation to locomotion. This study also measures the rate of hemocyte locomotion. Locomotory activity of mammalian leucocytes has been described with a variety of techniques (Wilkinson *et al.,* 1982; Keller, 1983) and Tackle (1984) recently utilized the technique of Lackie and Burns (1983) to describe insect hemocyte locomotion.

Results indicate that increased salinity retards hemocyte activity in both acute and acclimated conditions. The oysters in this study were also used to investigate the effects of different acclimation salinities on the physiological scope for growth (Newell *et al.,* 1986), and the protein and carbohydrate content of their cell-free hemolymph (Fisher and Newell, in prep.).

Materials and Methods

Oyster collection and acclimation

High-salinity oysters (HS) were collected from an oceanic location at Wachapreague, Virginia, and low-salinity oysters (LS) were dredged from the Tred Avon tributary of the Choptank River in the upper Chesapeake Bay. HS (mean dry weight ± S. D. = 1.50 ± 0.46 g) were collected on 21 May 1984 and LS (1.13 ± 0.31 g) had been collected one week earlier and held in flow-through water in our laboratory, directly across the Choptank River from the Tred Avon site. Salinities at Wachapreague ranged from 27–32 ppt during May while those in Tred Avon ranged from 6–8 ppt.

Oysters were initially held at field ambient 15°C, then raised to 21°C for the salinity adjustment and acclimation period. Salinity adjustments were made in 6 ppt stages after an interval of at least 3 days at each stage (Table I). Acclimation to 6, 12, 18, 24, 30, and 36 ppt salinity occurred in 6 separate aquaria held at 21°C with 12 oysters from each location at each salinity. Acclimation periods varied, but the total number of days in adjustment and acclimation was the same for oysters from each location at any given salinity (Table I).

Oysters were fed 500 ml algae twice a day (*Isochrysis aff. galbana* and *Thalassiosira pseudonana*) at concentrations about 3% of the oysters' dry weight per day. Fine silt (<63 μm) was also added to the algae to improve oyster feeding and the water was continuously aerated. Water was changed and salinity adjusted twice each week.

Solutions and withdrawal of hemolymph

Two concentrated stock solutions were made for these experiments. Full-complement water (FCW) consisted of salts added in the proportions that they would normally

TABLE I

Adjustment and acclimation schedule[1]

Acclimation salinity	Days at intermediate salinities		Days at acclimation salinity	
	HS	LS	HS	LS
6	12	0	32	44
12	9	0	26	35
18	5	5	19	19
24	0	8	29	21
30	0	11	38	27
36	0	14	41	27

[1] A schedule of the number of days that oysters at each acclimation salinity spent during salinity adjustment (intermediate salinities) and at the final acclimation salinity.

be found in sea water. This consisted of 0.034 g $NaHCO_3$, 12.23 g $MgSO_4 \cdot 7H_2O$, 8.99 g $MgCl_2 \cdot 6H_2O$, 2.61 g $CaCl_2 \cdot 2H_2O$, 1.30 g KCl, and 47.84 g NaCl per liter of deionized H_2O. This made an artificial sea water equivalent to 60 ppt salinity which was diluted with distilled water to the test salinity (osmotic pressure measured by "Osmette A", Precision Systems, Inc.). Calcium- and magnesium-free water (CMFW) excluded calcium and magnesium ($MgSO_4$, $MgCl_2$, and $CaCl_2$) and sulfate requirements were met by the addition of 7.04 gl^{-1} Na_2SO_4. This solution was also diluted to the appropriate salinities. Tris buffer (10 m*M*) was added to both FCW and CMFW after dilution and the pH adjusted to 7.40 ± .03 with HCl.

A 3 ml syringe was loaded with 1.5 ml CMFW + 2% EDTA (59.5 m*M* EDTA). The CMFW was diluted to the oyster acclimation salinity (=CMFW-iso, or FCW-iso for FCW adjusted to the oyster acclimation salinity). Oyster valves were notched on the posterior side and fluid in the mantle cavity was drained. The syringe needle (22G, 1–½") was inserted into the adductor muscle and hemolymph was slowly drawn into a 1:1 dilution with the CMFW-iso + 2% EDTA. The syringe was gently rocked three-times to mix the contents without creating hemocyte clusters and one drop was added to each side of a Neubauer hemocytometer for hemocyte counts (Stein, 1975). The syringe was gently rocked 5–10 more times to allow aggregation of hemocytes into small clusters.

Hemocyte function tests

Within 15 seconds of withdrawal, 2–3 drops of the diluted hemolymph were added to the wells of a plastic tissue culture dish which had been pre-loaded with 1 ml CMFW-iso. The hemocytes were allowed to settle for 5 minutes, then EDTA was washed from the medium three times by slowly withdrawing 0.9 ml from the well and replacing it with CMFW-iso. The final removal of medium left the hemocytes adhering to the bottom of the well covered by 0.1 ml CMFW-iso. The timed portion of the experiment began when FCW was added at salinities of 6, 12, 18, 24, 30, or 36 ppt. The hemocytes in each well were examined with a Nikon inverted microscope (200×) after 5, 10, 15, 30, 45, 60, 90, and 120 minutes. At each examination, hemocyte clusters were checked for ameboid cells forming along the periphery and starting to migrate from the cluster. The sample was marked positive (=TTS) when the majority of clusters had ameboid cells along the entire periphery. Hemocytes that did not spread by 120 minutes were assigned the value of 150 minutes because after this time the hemocytes would sometimes disaggregate without distinct spreading or locomotion.

The rate of locomotion (ROL) was measured for 10–20 ameboid granular hemocytes in selected wells. A video camera was used to project the microscope image onto a television screen and the paths of the hemocytes were traced for 5 minutes on clear plastic overlayed on the screen. Most tracings were made between 60 and 150 minutes after the experiment began and readings of less than 0.75 μm/min were discounted as non-mobile cells.

Statistical evaluation

A Student-Newman-Kuels test (SNK) was used to determine differences between means for TTS measurements. Analysis of variance (ANOVA) was used to determine collection site, acclimation salinity, and test salinity differences. Linear regression was employed to determine the slopes of ROL measurements *versus* test salinity by acclimation salinity and location (origin). Those acclimation salinities for each location that exhibited a slope significantly different from zero were pooled for analysis of covariance and a regression equation was determined from the pooled data.

Results

Hemocytes drawn into a 1:1 dilution with CMFW + 2% EDTA were spherical and non-mobile, but were not inhibited from adhering to each other and forming clusters on contact. The cells, whether single or in clusters, remained spherical and non-mobile during and after washing with CMFW. Only the addition of calcium and magnesium (as FCW) initiated spreading and locomotion by the hemocytes. Figure 1 shows the transformation and migration of granular hemocytes after the addition of FCW. EDTA was necessary because withdrawal of hemocytes in CMFW alone was not sufficient to keep all hemocytes spherical. Preliminary experiments had shown that withdrawal into CMFW + 2% EDTA, washing, and then adding FCW created a slightly longer TTS than withdrawal directly into FCW, but the additional steps had no measurable effect on ROL.

Time to hemocyte spreading (TTS)

Full-complement water, containing calcium and magnesium, was required for the initiation of cell spreading and subsequent mobility. There were two TTS responses to acute changes in salinity (Fig. 2). The most dramatic was a large increase in TTS when hemocytes from oysters acclimated to low salinities were raised to higher salinities. Greater differences between the acclimation and test salinity resulted in longer times to cell spreading. Hemocytes acclimated to 6 ppt and tested *in vitro* at 36 ppt, did not become ameboid after 120 minutes and were assigned the (conservative) value of 150 minutes. The second response to acute salinity change was a slight increase in TTS when hemocytes from oysters acclimated to high salinities were tested at the lowest test salinities. The shortest TTS resulted from hemocytes tested at or near their acclimation salinity and there were no significant differences in TTS among hemocytes tested and acclimated at the same salinities (circled points in Fig. 2). Oysters from the HS location had a faster TTS response than those from the LS location (ANOVA, $P < .05$).

Rate of hemocyte locomotion (ROL)

Granular hemocytes were not mobile when they were spherical, and the presence of calcium and magnesium was necessary for their transformation to an ameboid

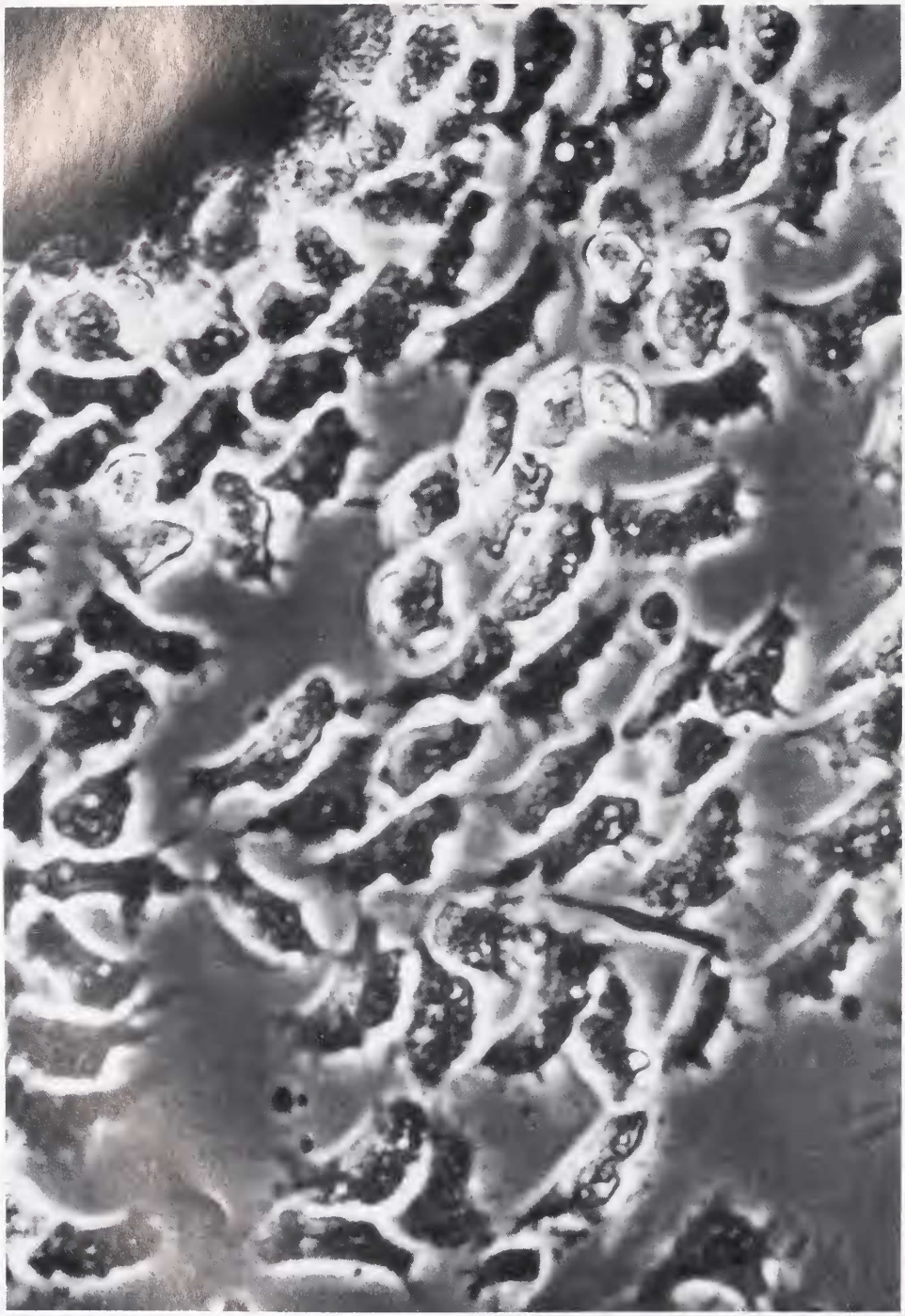

FIGURE 1. Oyster hemocytes on the periphery of a hemocyte cluster transform from a spherical to an ameboid shape (cell spreading) and migrate away from the cluster (locomotion). The crescent shape with the convex portion of the cell leading is typical for the locomotory hemocytes.

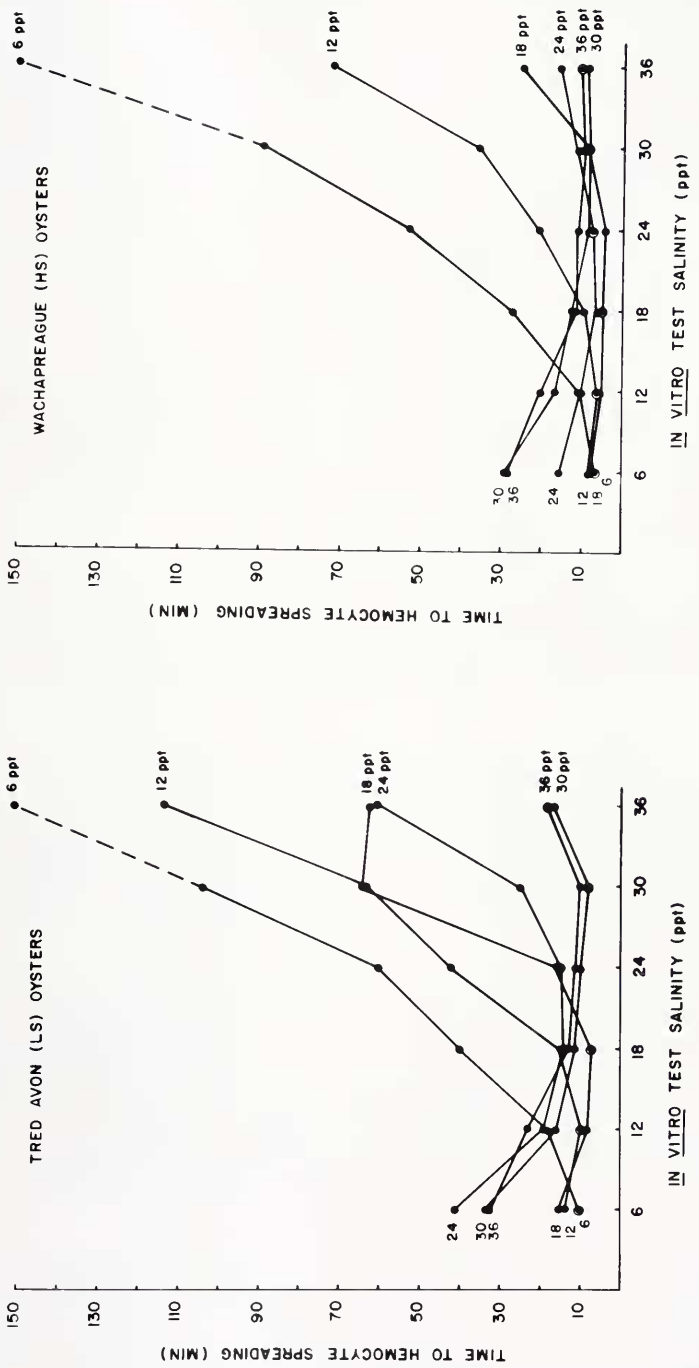

FIGURE 2. Average time to hemocyte spreading for oysters collected from a low-salinity (LS) and a high-salinity (HS) location and tested in a range (6–36 ppt) of salinities. Results from oysters at each acclimation salinity are presented separately. Dashed lines represent results obtained from assigned values (see text) and circled points represent measurements where the *in vitro* test salinity was the same as the acclimation salinity. Standard errors are presented in Table II.

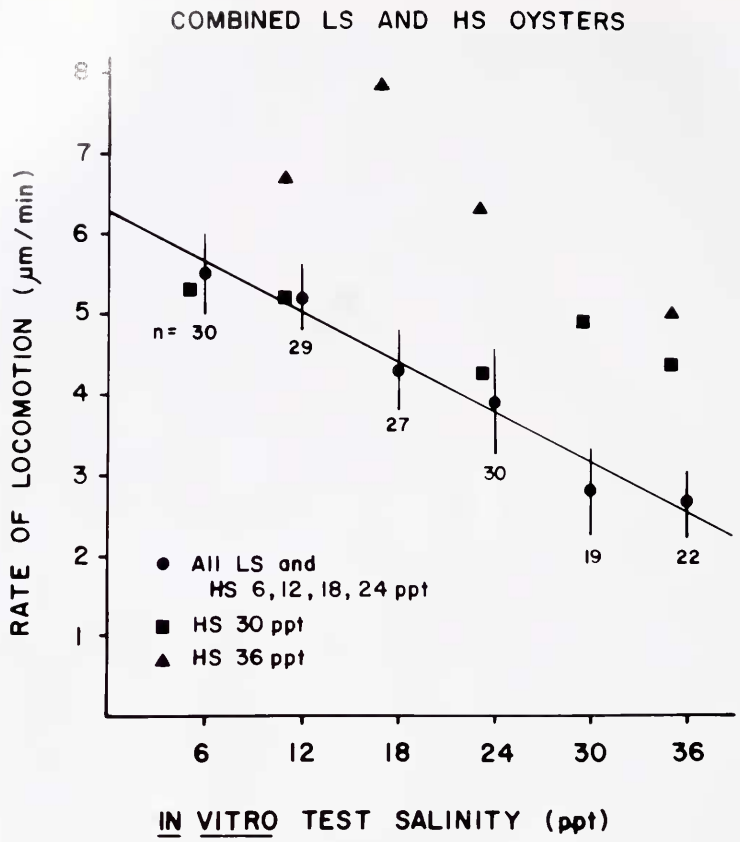

FIGURE 3. Average rates of hemocyte locomotion (ROL) for oysters collected from both locations and tested in a range (6–36 ppt) of salinities. Results from the high-salinity oysters acclimated to 30 and 36 ppt salinity (HS-30 and HS-36) did not exhibit slopes different from zero and are presented separately. All other oysters at all acclimation salinities from both collection sites showed slopes significantly different from zero and were pooled for presentation. There were no differences in the pooled data (ANCOVA, $P > .05$) and error bars show 95% confidence limits.

shape. Acute salinity changes affected the ROL of oyster hemocytes from both locations at nearly all acclimation salinities. That is, the ROL at all acclimation salinities from both locations, except HS oysters acclimated at 30 and 36 ppt, had a slope significantly different from zero when ROL was plotted *versus* test salinity (linear regression, $P < .05$). The results are presented in Figure 3, where the regression means and 95% confidence limits were pooled for data with no differences (ANOVA, $P > .05$), which excluded only HS-30 and HS-36. The regression is described by the equation

$$\text{ROL } (\mu\text{m/min}) = 6.25 - 0.104 \text{ test salinity (ppt)}$$

It is emphasized that this relationship holds for all LS oysters but only for HS oysters acclimated at 6–24 ppt.

There was a significant decrease in ROL for LS hemocytes acclimated and tested at 30 and 36 ppt (Fig. 4; SNK, $P < .05$). Thus, after 27 days of acclimation to high salinity, LS oyster hemocytes were still slower in high-salinity conditions than HS hemocytes. HS hemocytes showed no differences in ROL when measured at different

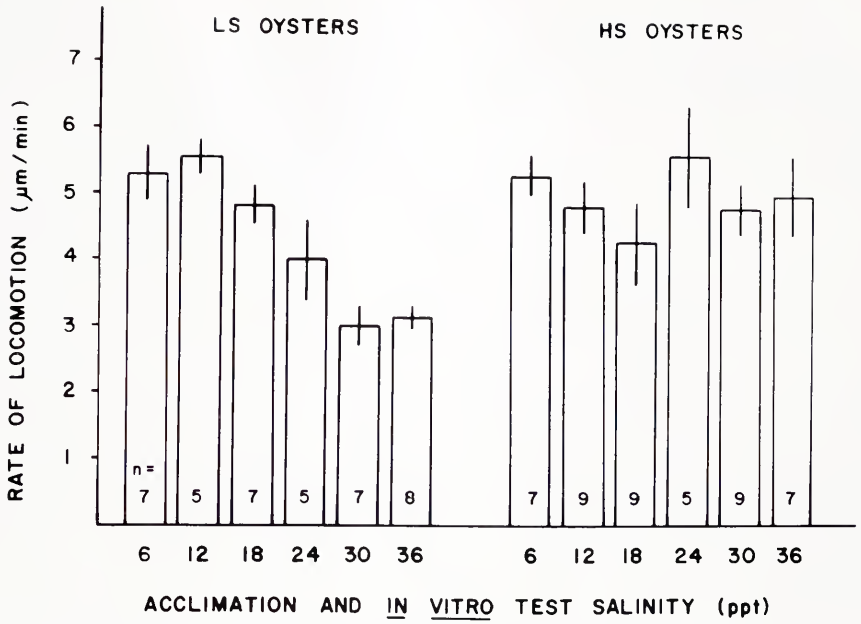

FIGURE 4. Average rates of hemocyte locomotion and standard errors for LS and HS of oysters in conditions where the *in vitro* test salinity was the same as the acclimation salinity. The periods of acclimation at each salinity ranged from 19–44 days (Table I). The LS oysters acclimated and tested at 30 and 36 ppt salinity were significantly slower than LS oysters from lower acclimation salinities while there were no differences between HS oysters from any acclimation salinity.

acclimation salinities (Fig. 4; ANOVA, $P > 0.05$). Hemocytes of oysters from both locations held and tested at their respective ambient salinities were not different (Fig. 4). The acute and acclimated TTS and ROL tests for both locations are compared in Table II. There were dramatic differences in TTS for both locations. TTS responses for LS oysters in higher salinities and for HS oysters in lower salinities had returned to ambient values after the acclimation period. ROL measurements for HS oysters showed no effect due to acute salinity reductions, and this did not change during the acclimation period. ROL measurements for LS oysters showed that hemocytes from 18 and 24 ppt salinities were partially returned to ambient rates, whereas hemocytes from 30 and 36 ppt salinities remained at the same reduced rates that were caused by the acute salinity increase.

Hemocytometer counts showed that there was no difference in the number of circulating granular hemocytes in the hemolymph of oysters from the two collection sites (Fig. 5), but there were greater numbers of circulating agranular hemocytes in the HS oysters at all acclimation salinities (ANOVA, $P < 0.05$).

DISCUSSION

Two major themes pursued by previous investigators have been the nutritive and defensive roles of oyster hemocytes (Feng *et al.*, 1977; Cheng, 1977). Phagocytosis is the major hemoctye activity that underlies these functions. Presumably, phagocytosis is influenced by the response-time of the hemocytes (TTS) and their subsequent locomotory ability (ROL). Phagocytosis may also be directly linked to locomotion by the process of endocytosis (Bretscher, 1984), by shared calcium-binding sites on the

TABLE II

Comparison of acute and acclimated conditions[1]

TTS: minutes (±S.E.)	Salinity					
	6	12	18	24	30	36
LS						
acute	†10.4 (0.5)	17.7 (3.6)	40.1 (4.3)	60.4 (8.4)	103.8 (10.2)	150.0 (0.0)
acclimated	†10.4 (0.5)	9.9 (1.0)	7.1 (3.5)	15.0 (4.7)	8.9 (2.4)	18.5 (3.0)
HS						
acute	28.9 (3.9)	17.0 (2.7)	12.7 (2.5)	8.4 (0.8)	†8.4 (0.8)	9.2 (0.6)
acclimated	7.6 (1.0	6.3 (1.7)	5.5 (0.5)	7.5 (1.4)	†8.4 (0.8)	10.9 (0.7)
ROL: μm min^{-1} (±S.E.)	Salinity					
	6	12	18	24	30	36
LS						
acute	†5.31 (.34)	5.00 (*)	3.38 (.38)	2.03 (.18)	3.13 (*)	2.51 (.18)
acclimated	†5.31 (.34)	5.56 (.26)	4.81 (.33)	4.02 (.61)	3.01 (.3)	3.16 (.17)
HS						
acute	5.25 (.31)	5.20 (.91)	4.38 (*)	4.26 (.35)	†4.83 (.41)	4.44 (.18)
acclimated	5.30 (.30)	4.83 (.39)	4.31 (.62)	5.58 (.77)	†4.83 (.41)	5.00 (.61)

[1] A comparison of time to hemocyte spreading (TTS) and rate of hemocyte locomotion (ROL) measurements in acute and acclimated conditions. Certain values (*) are estimated from the regression depicted in Figure 5. Acute data came from oysters in groups LS-6 and HS-30, the ambient salinities for Tred Avon and Wachapreague, respectively. Acclimated data came from tests at each acclimation salinity. Certain data (†) are repeated for acute and acclimated conditions.

cell membranes (Stockem and Klein, 1979), and by microfilament involvement (Spooner *et al.*, 1971; Axline and Reaven, 1974). Thus, TTS and ROL measure hemocyte characteristics fundamental to the phagocytic process. Although the effects of salinity on phagocytosis and overall defense must be further investigated, these hemocyte function tests may prove to be valuable indicators of the vitality of the oyster defense system.

The acute salinity tests revealed that hemocyte transformation was retarded by increased salinity (Fig. 2) and that greater retardation resulted from greater differences between acclimation and test salinities. This implies that the time period was being used by the hemocytes for osmotic adjustment. It is possible that hemocytes must be iso- or hyperosmotic to their environment in order to become ameboid. When *in vitro* measurements on oyster hemocytes were made at acclimation salinities, only 5–10 minutes were required for hemocyte spreading (circled points in Fig. 2). This may have been the fastest that spreading could occur, since no osmotic adjustment was necessary under such conditions.

The effect of salinity on hemocyte locomotion was evident in oysters from both locations (Fig. 5). As with TTS measurements, a greater salinity differential resulted in a greater change in hemocyte activity. Acute salinity increases retarded the activity of *C. virginica* hemocytes and acute salinity decreases enhanced hemocyte activities, implying that it is more time-consuming for oysters to adjust to higher salinities than to lower salinities. This also has been found in *Modiolus demissus,* where intracellular biochemical changes stabilize within hours in hypo-osmotic conditions (Pierce, 1970) but require weeks in hyperosmotic conditions (Baginski and Pierce, 1975). Several investigators have found that isolated cells respond more rapidly to resist swelling (hypo-osmotic conditions) than to resist shrinking (Davenport, 1982, for review).

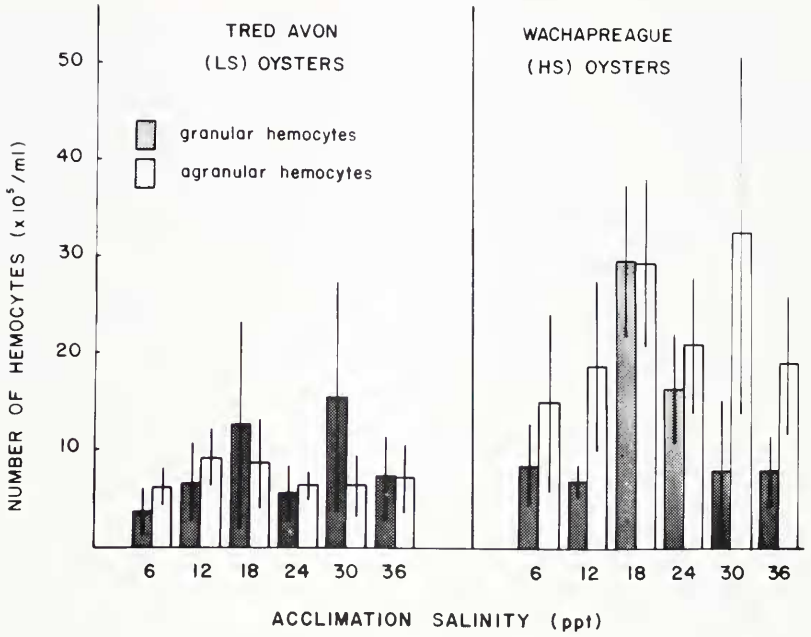

FIGURE 5. Average numbers and standard error bars for circulating granular and agranular oyster hemocytes for oysters collected from each location at each acclimation salinity. The number of agranular hemocytes is greater for oysters collected from a high-salinity (HS) location over all acclimation salinities.

Wachapreague oysters (HS) acclimated at 30 and 36 ppt, by maintaining the same hemocyte ROL at all test salinities, were exceptions to the ROL pattern (Fig. 3). Yet this rate was equal to the maximum attained by hemocytes under any other condition. The enhancement effect of lowered salinity may have been masked by the fact that they were already mobile at a maximum rate. Enhancement with decreased salinity for the other HS oyster hemocytes might then be an artificial response, brought about by their acclimation to lower salinities.

Hemocytes from Tred Avon (LS) oysters maintained at their ambient salinity (LS-6) showed dramatic TTS and ROL responses to acute increases in salinity (Figs. 2, 3). Measurement of TTS after high-salinity acclimation revealed that hemocyte transformation was no longer retarded by high salinity, *i.e.,* they were completely acclimated. Hemocyte locomotion (ROL), however, showed only partial acclimation to 18 and 24 ppt salinity, and was still retarded by 30 and 36 ppt salinity (Table II, Fig. 4). Acclimation by HS oysters is difficult to discern since the acute effect of decreased salinity was relatively minor in both TTS and ROL.

Since the retarded rate of locomotion for LS oysters in high salinities did not completely acclimate to ambient rates, it can be assumed that either the acclimation period was too short or that there were genetic differences between the LS and HS oysters. The latter possibility is reinforced by large differences in protein and carbohydrate concentrations in the cell-free plasma from the same oysters studied here (Fisher and Newell, in prep.), by differences in hemocyte counts (Fig. 5), and by differences in feeding rate, oxygen consumption and scope for growth which were also measured from the same oysters studied here (Newell *et al.,* 1986). Yet, electrophoretic comparisons (Newell *et al.,* 1986) could determine no dissimilarities between the populations.

There is an apparent contradiction between the evidence which showed a retarding influence of high salinity on hemocytes and the measurements made on HS oysters at their ambient salinities. Comparison of TTS and ROL for HS oysters at 30 and 36 ppt salinity with LS oysters at 6 ppt, indicates that high salinity does not have a detrimental effect in the field. A possible explanation for this discrepancy is that HS oysters have found a means to compensate for the high salinity through adaptation or long-term acclimation. High-salinity oysters in Delaware Bay that have increased their resistance to the MSX parasite through laboratory selection experiments (Haskin and Ford, 1979) may be an example of such an adaptation.

High-salinity susceptibility to diseases may be a consequence of such factors as intolerance of low salinities by the parasites (Haskin and Ford, 1982) and the downstream force and dilution of infective particles by fresh water (Mackin, 1955; Haskin and Ford, 1982). These results imply that salinity effects on the oyster defense system may also be a factor. For example, a decrease in hemocyte activity may have abetted the greater susceptibility to MSX shown by low-salinity oysters transplanted in Delaware Bay (Haskin and Ford, 1979). Similarly, the enhancement of hemocyte locomotion in reduced salinities may have aided in the expulsion of MSX from oysters transplanted to low salinity (Sprague *et al.*, 1969; Haskin and Ford, 1982).

The types or numbers of hemocytes available may also play a role in defense. Hemocyte counts in drawn hemolymph (Fig. 5) reflect only the circulating hemocyte level and it is unlikely that these circulating levels are an accurate estimate of the total hemocyte count (Feng, 1965). It is interesting however, that there is a greater number of circulating agranular hemocytes in HS oysters than in LS oysters. Farley (1968) found that these agranular hemocytes were present in much greater number in MSX-infected oysters. Only one oyster in this study was found to be infected with MSX and that animal had circulating granular and agranular hemocyte counts average for its group.

Measurement of hemocyte activity can aid in understanding cellular defense processes in bivalves. In this study, hemocyte measurements yielded sensitive and specific responses to salinity changes. Hemocyte spreading may be linked to intracellular volume regulation and the salinity effects on hemocyte locomotion may have illustrated a fundamental characteristic of ameboid locomotion. In addition, differences in hemocyte activities found between the low- and high-salinity collection sites raise questions concerning the long-term effects of adaptation to different salinities. Since the HS oysters were survivors in a location with heavy disease pressure, it is intriguing to speculate whether their ability to maintain maximal hemocyte activity in high salinities provided them with greater disease resistance.

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